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# Characterization of H9N2 avian influenza viruses from the Middle East demonstrates heterogeneity at amino acid position 226 in the hemagglutinin and potential for transmission to mammals

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ABSTRACT

Next-generation sequencing (NGS) technologies are a valuable tool to monitor changes in viral genomes and determine the genetic heterogeneity of viruses. In this study, NGS was applied to clinical poultry samples from Jordan to detect eleven H9N2 low pathogenic avian influenza viruses (LPAIV). All of the viruses tested belonged to Middle East A genetic group of G1 lineage. Deep sequencing demonstrated a high degree of heterogeneity of glutamine and leucine residues at position 226 in the hemagglutinin (HA) gene, which increases specificity to either avian or mammalian-type receptors. Moreover, additional amino acid changes in PB1, PA, M1, M2, and NS1 were identified among the viruses tested. Compared to single gene amplification, application of NGS for surveillance and characterization of H9N2 LPAIV provides a complete genetic profile of emerging isolates and better understanding of the potential of zoonotic transmissions to mammals.

## 1. Introduction

Avian influenza virus (AIV) belongs to the family Orthomyxoviridae and have a segmented, single-stranded, negative sense RNA genome with enveloped virions. The 13.5 kb genome consists of eight segments coding 14 known proteins: the polymerase proteins (PB1, PB2, PA) (Chen W1, 2001; Wise et al., 2009), nucleocapsid protein (NP), hemagglutinin (HA), neuraminidase (NA), matrix proteins (M1 and M2), nonstructural proteins (NS1 and NS2) and PB1-F2, PB1 N40, PA-X and M42 proteins (Jagger et al., 2012; Wise et al., 2009, 2012). Influenza viruses are categorized based on their two surface antigens, HA (16 subtypes, H1-H16), and NA (N1 to N9). Based on the pathogenicity AIV can be divided into the low and high pathogenicity avian influenza viruses.

Among low pathogenicity AIV (LPAIV), Eurasian H9N2 have been endemic in domestic poultry population across North Africa, the Middle East and Asia since it emerged in China during 1994 (Zhang et al., 2009). Phylogenetic and antigenic analysis have divided H9N2 LPAIV Eurasian strains into the G1 lineage, the Y280 lineage, and the Y439/Korean lineage (Guan et al., 1999; Lee and Song, 2013; Matrosovich et al., 2001). These viruses are responsible for severe economic losses due to declined egg production and moderate to high mortality in broiler-type chickens (Guo et al., 2000; Lee et al., 2007; Wu et al., 2008). In addition, H9N2 LPAIV has caused repeated human infections in Asia since 1998, raising public concerns about increasing pandemic potential (Butt et al., 2005; Lin et al., 2000; Matrosovich et al., 2001). These sporadic cases of human infections have been associated with some specific genetic changes, including the change of amino acid at position 226 from glutamine (Q) to leucine (L) in the HA receptor binding site (RBS) which plays a key role in human virus-like receptor specificity and promotes the transmission of H9N2 in ferrets (Wan and Perez, 2007).

H9N2 LPAIV isolated from Central Asia and the Middle East between 1998 and 2010 can be genetically divided in four distinct groups (A-D). Groups A and B have been found in Central Asia and the Middle East, whereas groups C and D were detected in United Arabic Emirates between 2000 and 2002 and in Iran from 1998 to 2007 (Fusaro et al., 2011). This subtype has been isolated from mammals and evolved through point mutations and reassortment events between H9N2 viruses and other influenza virus subtypes (Chaudhry et al., 2015; Pu et al., 2015; Sun et al., 2015). Therefore, surveillance and characterization of viruses are essential to better understand any continuing public health risk in this area. Recent studies reported the evolutionary history and current situation of G1-lineage H9N2 in Iraq and Pakistan (Kraidi et al., 2016; Lee et al., 2016), but limited information and genetic sequence of H9N2 LPAIV from Jordan are available. In Jordan, the H9N2 virus was first reported from chickens and domestic ducks in 2003 (Monne et al., 2007). A high prevalence of H9 subtype was found

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**Table 1**
The frequency of the nucleotide changes at position 226 in HA gene sequence from avian influenza isolates obtained in these studies.

| H9N2 strain | Variant frequency (%) | | Q226L |
|---|---|---|---|
| | CAG (Q) | CTG (L) | |
| A/chicken/Jordan/12/2003 | 1815 (99.5) | 1 (0.1) | Q |
| A/chicken/Jordan/11/2003 | 244 (98.6) | 2 (0.9) | Q |
| A/chicken/Jordan/56/2003 | 4053 (99.9) | 0 (-) | Q |
| A/chicken/Jordan/47/2003 | 722 (89.3) | 82 (10.2) | Q |
| A/chicken/Jordan/14/2003 | 223 (14.6) | 1360 (85.4) | L |
| A/chicken/Jordan/13/2003 | 1864 (66.3) | 938 (33.4) | Q |
| A/chicken/Jordan/10/2003 | 4861 (99.3) | 26 (0.6) | Q |
| A/chicken/Jordan/45/2004 | 334 (94.6) | 17 (4.8) | Q |
| A/chicken/Jordan/70/2004 | 913 (33.9) | 1778 (65.9) | L |
| A/chicken/Jordan/55/2004 | 5 (0.1) | 2633 (99.9) | L |
| A/chicken/Jordan/88/2005 | 2480 (56.3) | 1914 (43.4) | Q |

in broiler and layer flocks in Jordan confirming endemic nature of this subtype in Jordan (Roussan et al., 2009).

In this study, Next-generation sequencing (NGS) was applied to poultry diagnostic field samples and the complete genomes of eleven H9N2 LPAIV isolates were characterized for genetic features of these viruses. NGS revealed the presence of viral subpopulation with mutations at numerous sites in the viral genome associated with host tropism for both avian and mammalian species.

## 2. Material and methods

### 2.1. Viruses

Oral swab samples were collected in Jordan poultry flocks between 2003 and 2005. The list of viruses are shown in Table 1. Samples were passaged in 9–11 day of specific pathogen free embryonating chickens eggs by standard methods (Senne, 2008). Following three days of growth the allantoic fluid was harvested. Nucleotide sequences of the H9N2 LPAIVs identified here have been deposited in GenBank under accession numbers: A/chicken/Jordan/12/2003 (ch/12/03) (MF673340- MF673347), A/chicken/Jordan/11/2003 (ch/11/03) (MF673380-MF673387), A/chicken/Jordan/56/2003 (ch/56/03) (MF673348- MF673355), A/chicken/Jordan/47/2003 (ch/47/03) (MF673372-MF673379), A/chicken/Jordan/14/2003 (ch/14/03) (MF673356-MF673363), A/chicken/Jordan/13/2003 (ch/13/03) (MF673316- MF673323), A/chicken/Jordan/10/2003 (ch/10/03) (MF673364-MF673371), A/chicken/Jordan/45/2004 (ch/45/04) (MF673324-MF673331), A/chicken/Jordan/70/2004 (ch/70/04) (MF673308- MF673315), A/chicken/Jordan/55/2004 (ch/55/04) (MF673300- MF673307), A/chicken/Jordan/88/2005 (ch/88/05) (MF673332- MF673339).

### 2.2. RT-PCR

RNA extraction was performed using RNeasy Mini Kit (QIAGEN, Valencia, USA). One step RT-PCR was conducted with 5 µl of RNA template in a final reaction volume of 50 µl using OneTaq® One-Step RT-PCR Kit (NEB) with the primers Optil-F1 5`-GTT ACG CGC CAG CAA AAG CAG G-3`, Optil-F2 5`-GTT ACG CGC CAG CGA AAG CAG G-3`, and Optil-R1 5`-GTT ACG CGC CAG TAG AAA CAA GG-3`. The PCR cycling was performed as follows: 95 °C for 2 min, 42 °C for 60 min, 94 °C for 2 min, 5 cycles of 94 °C for 30 s, 44 °C for 30 s, and 68 °C for 3.5 min, followed by 26 cycles of 94 °C for 30 s, 57 °C for 30 s, 68 °C for 3.5 min with a final extension at 68 °C for 10 min.

### 2.3. Genome sequencing

The Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and 0.2 ng/µl (1 ng total) of ds cDNA were used in this study to generate multiplexed paired-end sequencing libraries, according to the manufacturer's instructions, as previously described (Chrzastek et al., 2017). The ds DNA was fragmented and tagged with adapters by Nextera XT transposase. The Nextera XT transposome fragmented PCR amplicons with added adaptor sequences enabled a 12-cycle PCR amplification to append additional unique dual index (i7 and i5) sequences at the end of each fragmented DNA for cluster formation. PCR fragments were purified on Agencourt AMpure XP beads (Beckman Coulter). Fragments were analyzed on a High Sensitivity DNA Chip on the Bioanalyzer (Agilent Technologies) before loading on the sequencing chip. The barcoded multiplexed library sequencing was performed on an Illumina MiSeq (Illumina). The *de novo* and directed assembly of genome sequences were performed using the GALAXY software (Afgan et al., 2016; Dimitrov et al., 2017) and Geneious 9.1.2 (Kearse et al., 2012), respectively. The reads were directly mapped to A/chicken/Israel/1808/2004(H9N2) reference genome (8 segments, GenBank accession no. EF492425, EF492396, EF492367, EF492327, EF492298, EF492265, EF492242, DQ683044).

### 2.4. Phylogenetic and SNP Analysis

Complete coding sequences of HA gene segment were used for comparative genetic analyses. The maximum likelihood (ML) phylogenetic tree of Asian H9N2 viruses and Bayesian relaxed clock phylogenetic tree of Mideast group A were constructed using nucleotide sequences. The nucleotide sequences of HA segment were aligned using MUSCLE (Edgar, 2004). For ML phylogenetic analysis, all full-length HA segment of Asian H9N2 available in the GenBank (n = 1706) were retrieved and representative sequences (n = 297) were selected based on sequence identity at 98% level using CD-HIT package (Huang et al., 2010). ML phylogeny of 22 Jordanian H9N2 with representative sequences was generated with RAxML using the GTR nucleotide substitution model, with among-site rate variation modeled using a discrete gamma distribution. Bootstrap support values were generated using 1000 rapid bootstrap replicates. To determine lineages and genotypes of H9N2 viruses, ML phylogenies of the other 7 AIV segments were also constructed. Bayesian relaxed clock phylogenetic analysis of Mideast group A (ntax = 54) was done using BEAST v1.8.3 (Drummond and Rambaut, 2007). We applied an uncorrelated lognormal distribution relaxed clock method, the HKY nucleotide substitution model and the Bayesian skyline coalescent prior. A Markov Chain Monte Carlo (MCMC) method to sample trees and evolutionary parameters was run for $1.0 \times 10^7$ generations. At least three independent chains were combined to ensure adequate sampling of the posterior distribution of trees. BEAST output was analyzed with TRACER v1.4 with 10% burn-in. A maximum clade credibility (MCC) tree was generated for each data set using TreeAnnotator in BEAST. FigTree 1.4.2 (http://tree.bio.ed.ac.uk/) was used for visualization of trees. Complete coding regions were aligned and used for subsequent single-nucleotide polymorphisms (SNP) analysis using Geneious v9.1.2 program.

## 3. Results

### 3.1. Genome sequencing of H9N2 isolates

All eight gene segments of the Jordanian H9N2 viruses were sequenced by high-throughput sequencing using the MiSeq platform and the reads covered 100% of the total genomes. Mapping of the reads to the reference genome (A/chicken/Israel/1808/2004(H9N2)) for all viruses was performed at an average mean depth of 5089.6 (Min. 1349, Max. 15993) for PB2, 2038.9 (Min. 882.3, max. 3027.8) for PB1, 7760 (Min. 1640.1, Max. 17200) for PA, 2274.2 (Min. 620, Max. 4143.8) for HA, 1636.2 (Min. 66.5, Max. 3600.1) for NP, 22209.5 (Min. 832.2, Max. 47373.3) for M and 21209.6 (Min. 2441, Max. 37092.2) for NS segment.

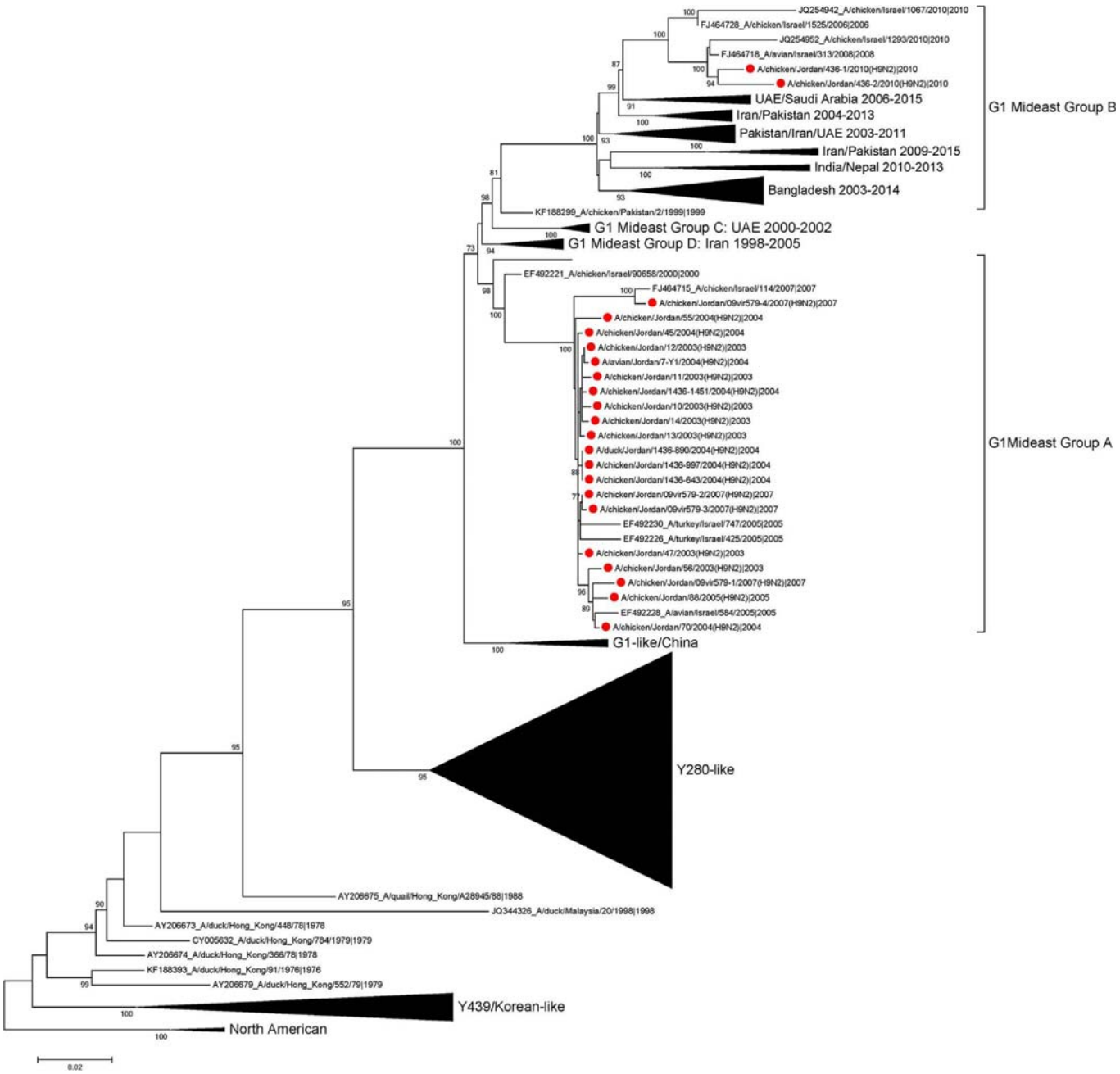


**Fig. 1.** Relaxed clock molecular phylogenetic tree for the hemagglutinin of H9N2 Jordanian viruses. All Jordanian sequences from GenBank and provided here are highlighted in red. The phylogenetic relationships and the evolutionary history have been estimated by molecular clock analysis.

### 3.2. Phylogenetic analysis

All of the isolates tested were LPAIV based on amino acid sequence at the HA proteolytic cleavage site (PARSSR/G). The homology analysis of the eleven viral genome sequences demonstrated that the HA genes shared very high nucleotide sequence identity of 98.5–99.8%. All eleven viral isolates obtained in Jordan from 2003 to 2005 belonged to Mideast group A and clustered with H9N2 viruses identified in Israel between 2000 and 2007 but differ from Jordanian H9N2 isolates identified in 2010 that belonged to Mideast group B (Fig. 1). Consistent with a previous study, (Fusaro et al., 2011), all eight genes of H9N2 Jordanian viruses collected from 2003 to 2005 were clustered with Mideast group A viruses whereas H9N2 Jordanian isolates identified in 2010 were clustered with Mideast group B viruses (Suppl. Fig. 1).

### 3.3. Non-synonymous mutations in the HA protein

The sequence analysis demonstrated genetically similar H9N2 Jordanian viruses that belong to genetic group A and carry non-synonymous changes in the protein coding regions of HA gene (Fig. 2). In particular, the frequency of the nucleotide changes in individual clones at position 226 in the HA gene sequence are shown in Table 1. Sequence analysis demonstrated that 3 of the isolates had L at position 226 in the HA (H3 numbering). In ch/13/03, the variation at amino acid residue 226 represent 66.4% of CAG (226 Q) and 33.5% CTG (226 L). In ch/88/05, the frequency of the codon was 56.3% of CAG (226 Q) and 43.4% of CTG (226 L). In ch/70/04, the frequency of the codon was 65.9% of CTG (226 L) and 33.9% of CAG (226 Q). The polymorphism analysis of the hemagglutinin gene of H9N2 Jordanian viruses is shown in Suppl. Fig. 2.

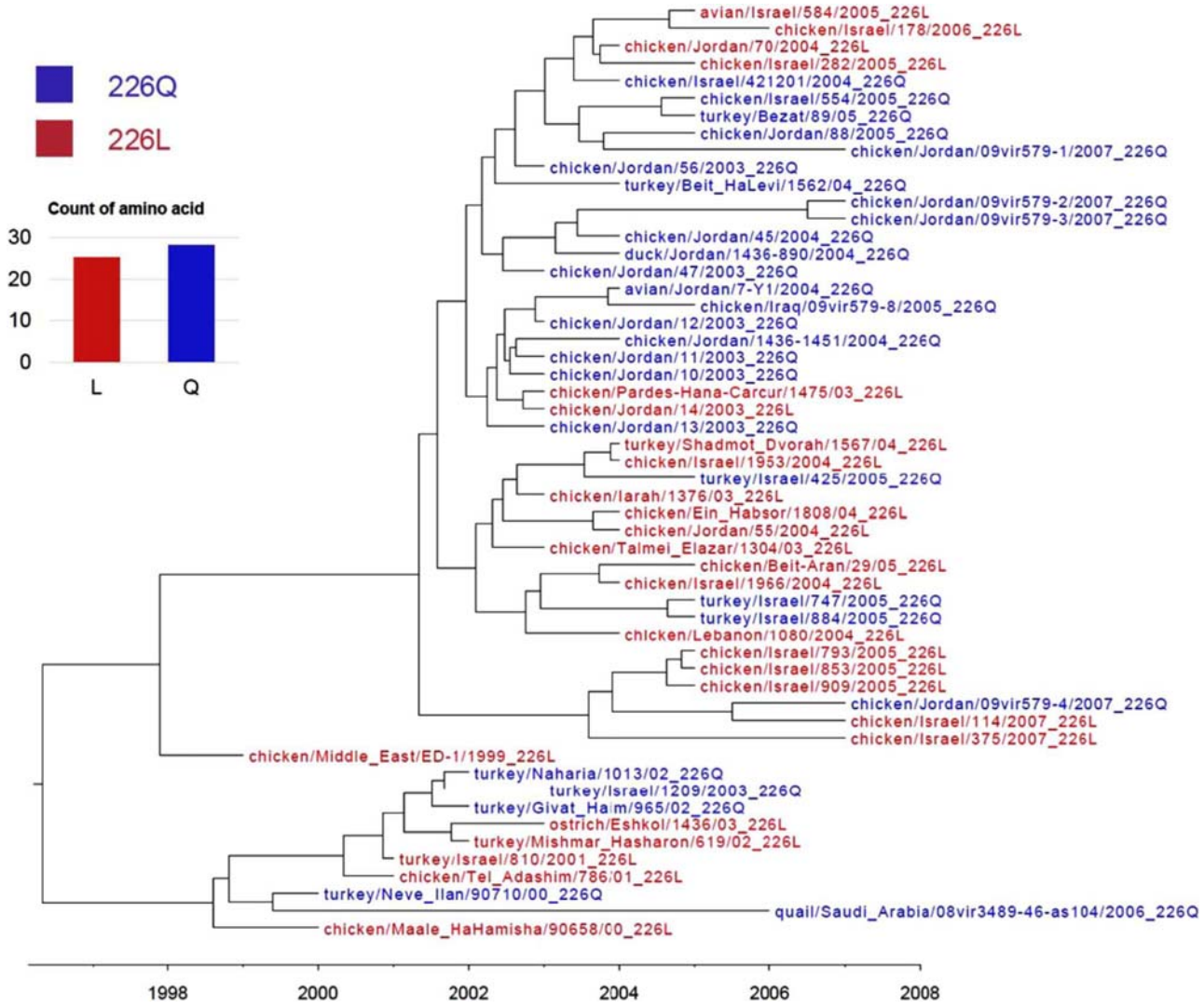


**Fig. 2.** Bayesian relaxed clock phylogenetic tree for the hemagglutinin of H9N2 Mideast group A with mixed populations at position 226. A 226Q and 226 L are highlighted in blue and red, respectively.

### *3.4. Other non-synonymous substitutions*

Amino acid substitutions identified from Jordan H9N2 viruses related to the increased replication or virulence of avian influenza viruses in mammals are shown in Table 2. The amino acid changes R207K, H436Y, and M677T in basic polymerase 1 (PB1), A515T in acidic polymerase (PA), N30D and T215A in matrix protein (M1), and P42S in nonstructural protein 1 (NS1) were conserved in all strains. Moreover, we have found V15I substitution on M1 protein, L55F on M2 protein, and the C-terminus of PDZ ligand motif in NS1 protein (EPEV) in all of the viruses tested. All viruses tested carry M106I mutation in NS1 except one isolate which contained a M106I and F103L mutation in NS1 segment. A detailed comparison of the amino acid differences among the H9N2 viruses demonstrated all of the H9N2 viruses have the amino acid 627 V in their PB2 which is rarely found in natural influenza isolates. The K339T substitution in PB2 subunit cap-binding pocket of influenza A virus was not observed among the Jordanian isolates; however, two isolates contain 339Q substitution in their PB2. In segment 6 (NA), the mutations that confer resistance to zanamivir, oseltamivir and/or peramivir were not detected. The amantadine and rimantadine resistance-conferring mutation S31N/G in M2 was not detected among the Jordanian H9N2 isolates.

## 4. Discussion

AIV H9N2 subtype has been endemic in poultry populations throughout Asia, the Middle East, Europe, and Africa since 1997 (Fusaro et al., 2011; Lee and Song, 2013). The viruses of G1 lineage are grouped into A and B genetic clades which predominant in the Middle East and have been circulating in this area since their first detection in 1998 − 1999 in Iran and Pakistan (Naeem et al., 1999; Nili and Asasi, 2003). The Jordanian H9N2 viruses shared close nucleotide homology with G1 lineage isolates from birds in Israel between 2000 and 2007. All H9N2 Jordanian viruses collected from 2003 to 2005 tested in this study belonged to Mideast group A whereas two H9N2 Jordanian isolates identified in 2010 belonged to Mideast group B (Fusaro et al., 2011). These two H9N2 viruses of Mideast group B identified in 2010 are the most recent H9N2 viruses reported from Jordan. Since limited information and genetic sequence of H9N2 LPAIV from Jordan are available after 2010, the current situation of H9N2 LPAIV in this area remains uncertain.

The recognition of appropriate forms of sialic acid (SA) by the HA protein is one of the critical barriers for AIV to replicate in different hosts (Wan and Perez, 2007). Avian influenza viruses preferentially bind alpha 2,3 linked SA(SA2,3 Gal) whereas mammalian influenza viruses preferentially bind in the 2,6 linkage (SA2,6 Gal). The amino

**Table 2**
Amino acid substitutions identified from Jordan H9N2 viruses isolated in these studies related to the increased replication or virulence of avian influenza viruses in mammals and reduced susceptibility to antiviral drugs.

| Gene | Substitution | Function | A/ch/Jo/12/2003 | A/ch/Jo/11/2003 | A/ch/Jo/56/2003 | A/ch/Jo/47/2003 | A/ch/Jo/14/2003 | A/ch/Jo/13/2003 | A/ch/Jo/10/2003 | A/ch/Jo/45/2004 | A/ch/Jo/70/2004 | A/ch/Jo/55/2004 | A/ch/Jo/88/2005 | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PB2** | E627K | virulence and transmission of H5N1 in mammals; enhanced polymerase activity; mammalian host adaptation | V | V | V | V | V | V | V | V | V | V | V | (Fan et al., 2014; Taft et al., 2015) |
| | D701N | | D | D | D | D | D | D | D | D | D | D | D | |
| | K147T | polymerase activity in mammalian cells of avian H5N1 mammalian cells; polymerase activity and virulence by regulating the cap binding activity | I | I | I | I | I | I | I | I | V | I | V | |
| | M147L | | | | | | | | | | | | | |
| | K339T | | K | K | K | K | K | K | K | K | Q | K | Q | |
| | A588T/I | | A | A | A | A | A | A | A | A | A | A | A | |
| **PB1** | R207K | polymerase activity in mammalian cells | K | K | K | K | K | K | K | K | K | K | K | (Hulse-Post et al., 2007) |
| | H436Y | polymerase activity and virulence in mallards, ferrets and mice | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | |
| | M677T | virulence-related mutation | T | T | T | T | T | T | T | T | T | T | T | |
| **PA** | A515T | polymerase activity in mammalian cells | T | T | T | T | T | T | T | T | T | T | T | (Li et al., 2011) |
| **HA**[a] | Q226L | promote the affinity of avian influenza viruses | Q | Q | Q | Q | **L** | Q | Q | **L** | **L** | Q | Q | (Guo et al., 2000; Matrosovich et al., 2001; Wan and Perez, 2007; Watanabe et al., 2011) |
| | I155T | for human-type receptors | T | T | T | T | T | T | T | T | T | T | T | |
| | H183N | | H | H | H | H | H | H | H | H | H | H | H | |
| | A190V | | A | E | V | A | A | A | A | A | A | A | A | |
| **NA** | 49–68 deletion[b] | Enhance virulence in mouse | Not found | | | | | | | | | | | (Matsuoka et al., 2009) |
| | V96A (116[c]) | Reduced susceptibility to zanamivir, oseltamivir and/or peramivir | V | V | V | V | V | V | V | V | V | V | V | (Govorkova et al., 2013; Hurt et al., 2012; Matrosovich et al., 2001; Oh and Hurt, 2014) |
| | E99A/G/V (119 [c]) | | E | E | E | E | E | E | E | E | E | E | E | |
| | I203M/V/L/K/R (221[c]) | | I | I | I | I | I | I | I | I | I | I | I | |
| | H254Y/R (274 [c]) | | H | H | H | H | H | H | H | H | H | H | H | |
| **M1** | N30D | mammalian host specific markers, virulence-related | D | D | D | D | D | D | D | D | D | D | D | (Fan et al., 2009; Senne, 2008; Wu et al., 2008) |
| | T139A | | T | T | T | T | T | T | T | T | T | T | T | |
| | T215A | | A | A | A | A | A | A | A | A | A | A | A | |
| | V15I | mammalian host specific markers; common substitution in H5N1 exhibiting high virulence in mice | I | I | I | I | I | I | I | I | I | I | I | |
| **M2** | L55F | mammalian host specific markers, virulence-related | F | F | F | F | F | F | F | F | F | F | F | (Lee et al., 2007; Pan and Jiang, 2009) |
| | S31N/G | amantadine and rimantadine resistance | S | S | S | S | S | S | S | S | S | S | S | |
| **NS1** | P42S | increased virulence in mice | S | S | S | S | S | S | S | S | S | S | S | (Dankar et al., 2013; Dankar et al., 2011; Jackson et al., 2008) |
| | F103L | | F | F | F | F | F | F | L | F | F | F | F | |
| | M106I | | I | I | I | I | I | I | I | I | I | I | I | |
| | PL motif | virulence-related (ESEV, EPEV, or KSEV) | EPEV | EPEV | EPEV | EPEV | EPEV | EPEV | EPEV | EPEV | EPEV | EPEV | EPEV | |

[a] H3 numbering
[b] The numbering of the NA deletion is relative to A/goose/Guangdong/1/1996
[c] amino acid position in N2

acid sequence analysis of all avian influenza A viruses of the H9N2 subtype identified in humans showed that 17 out of 21 viruses carried Q226L substitution in their HA gene (Suppl. Table 1). Futheremore, H9N2 AIVs found in other mammalian species (horse, dog, and mink), also carried Q226L substitution in HA gene confirming preferential binding of H9N2 viruses to mammalian receptors with SSA2,6 Gal. In contrast, the H9N2 AIV isolates found in pigs carried both, 226Q (54.5%) and 226 L (43.2%) variants in theirs HA segments (Suppl. Table 1), which matched the presence of both types of SA receptors in pigs (Ma et al., 2008). Comparison of genetic group A H9N2 viruses found in public databases along with H9N2 Jordanian isolated from 1999 to 2007 demonstrated mixed populations at position 226 in HA gene. However, the majority of recent H9N2 viruses in carry amino acid L226 in HA gene (Heidari et al., 2016; Kraidi et al., 2016; Lee et al., 2016a). These results suggest that the G1 Mideast H9N2 population of viruses have diversified over the past decade with a majority of recent isolates preferentially containing the ability to bind the SA2,6 Gal receptor. Traditional Sanger sequencing or NGS data without SNP analysis does not determine the genetic heterogeneity of viruses and therefore report only the major population of virus sequence per nucleotide location. The sequence data presented here demonstrate the presence of minor populations of H9N2 viruses contained with field samples that can preferentially bind to both avian or mammalian receptors and amy help explain the incidence of H9N2 avian influenza in humans from the Middle East.

The G1 lineage H9N2 LPAIV has been sporadically isolated from humans suffering respiratory illness (Lin et al., 2000). The adaptive changes that occurred in viral segments other than HA segment in Middle East may also pose a risk of interspecies transmission of H9N2 AIVs between poultry and mammals. Several other amino acid changes related to the increased replication or virulence of avian influenza viruses in mammals were identified in these Jordanian H9N2 viruses. In PB2 segment, all eleven H9N2 isolates tested contain 627 V but only two contain 339Q substitution, which is rarely found in natural influenza isolates. Taft et al. demonstrate that the replicative ability and virulence of A/Muscovy duck/Vietnam/TY93/2007 (H5N1) encoding PB2–627V was increased in mice compared with PB2–627E and were comparable to that of the virus encoding the mammalian-adapting PB2–627K residue (Taft et al., 2015). Futheremore, Fan et al. have demonstrated that three residues at position 147 T, 339 T and 588 T in PB2 play critical roles in the virulence of avian H5N1 influenza viruses in a mammalian host (Fan et al., 2014). Viruses with these three residues have been isolated from a lethal human case, and are more pathogenic than viruses with only the three residues or 627 K in PB2. In our study, eight viruses contained the 147I, 339 K, 588 A and 627 V in their PB2 segment which may suggest increased possibility to replicate in mammals. Moreover, the analysis demonstrated amino acid changes of R207K, H436Y, and M677T in PB1, A515T in PA, V15I, N30D and T215A in M1, L55F in M2 and P42S in the C-terminus of PDZ ligand motif EPEV in NS1, correspond to increased replication and virulence and were conserved among all the viruses tested (Fan et al., 2009). Interestingly, we have found that all the Jordanian viruses had M106I mutation in NS1 and one isolate had F103L and M106I mutation in NS1 segment which is related to increased Iinterferon antagonism and interstitial pneumonia in H5N1 HPAI infected mice (Dankar et al., 2013).

In conclusion, all H9N2 Jordanian viruses collected from 2003 to 2005 belonged to Mideast group A and carried many SNPs, including Q226L substitution in HA gene that contribute to increased replication and virulence in mammals. The identification of field samples containing mixed populations of H9N2 LPAI with mutations at amino acid position 226 in their HA segments suggests an ability to transmit into either species the virus may come into contact. These samples also contain additional qualities that would enhance replication and virulence in mamalian hosts. Due to reassortment events between H9N2 virus and other influenza virus subtypes, the surveillance and characterization of viruses is essential to better understand any continuing zoonotic public health risk.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2018.02.016.

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